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# Recent Advances In Technics For The Study Of Viruses

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# Recent Advances In Technics For The Study Of Viruses

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THE existence of viruses was suspected by Pasteur but it was not until 1897 that active work was begun on these agents. At this time Loeffler and Foesche demonstrated the existence of the virus of foot and mouth disease. Early workers depended upon the filter for the isolation of viruses and an attempt was made to grow viruses in artificial tissue cultures. Diagnostic tests were dependent upon the complement-fixation test and animal protection and neutralization tests.

Recent advances in the study of the viruses began with the introduction of the living chick embryo as a culture medium for isolation and propagation. Woodruff and Goodpasture (1) in 1931 established the virus of fowl pox upon the chorioallantoic membrane of chick embryos. These workers also demonstrated the value of the chick embryo for the production of vaccines.

Scientific advances depend upon the development of new tools and methods. Progress in the study of virus diseases was dependent upon progress in the fields of chemistry and physics. The approach taken in the field of physical chemistry has contributed many of the tools as well as much knowledge of the nature of viruses. The electron microscope, ultra-filter, and ultra-centrifuge are among the best known tools. Recently, the bacteriophage has been added to the list of new tools. These viruses of bacteria are being studied in detail in an effort to learn more of the biology of the animal viruses. Bacteriophage has been termed "virus technicians."

Filtration of tissue suspensions to isolate viruses has become of less importance as other tools are developed. Filters adsorb much of the virus and are awkward to use. Too often, a virus has been lost rather than isolated by this method. Its place has been taken by the centrifuge. Tissue suspensions centrifuged for 5 minutes at 1,500 r.p.m. are very satisfactory, the virus remaining in the supernatant. Chemically unstable viruses are more readily obtained by using this technic. Recently, antibiotics have been used to reduce bacterial contamination which often occurs in tissue containing virus. Penicillin, streptomycin, and the sulfonamides may all be used as they do not affect the viruses.

The chick embryo is widely used for the isolation, propagation, and diagnosis of the viruses. The embryos are easy to use, less bacterial contamination is encountered, and most of the animal viruses grow in the embryo fluids or upon the membranes.

Eggs to be used for the cultivation of viruses are incubated at 38° C. They are candled and marked for drilling. The shell is disinfected with alcohol and one or more holes drilled in the shell with a small drill. The area around the holes is painted with iodine and the eggs are ready for inoculation. The route of inoculation varies with the purpose for which the embryonating eggs are used.

The yolk sac inoculation is preferably used for the isolation of viruses. Admin-

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istration of viruses by way of the yolk sac results in a high per cent of infection of the embryos, membranes, and extra-embryonic fluids. A 5-day-old embryo is inoculated with 0.1 ml. to 1.0 ml. of a virus suspension through a hole in the shell over the air sac. A 10 ml. syringe with a 1.25 inch 20-gauge needle is used. In an alternate method, the inoculum is introduced through a hole in the shell opposite the embryo.

The chorio-allantoic route of inoculation is used primarily for the propagation of viruses. It has been found of special value in maintaining serial passage of viruses. Pathologic changes on the membrane are of diagnostic value and of use in titrating the pox virus. Using an 11- or 12-day-old embryo, 2 holes are drilled in the shell, one over the air sac, the other on the side. The chorio-allantoic membrane is drawn away from the egg shell by means of suction applied to the hole over the air sac. The inoculum is dropped upon the exposed membrane or is inoculated into the chorio-allantoic sac with a 27-gauge needle.

Two other routes of inoculation, intravenous and intraembryonic, are used in research studies. An 11- or 12-day-old embryo is used. The virus of rabies may be adapted to the chick embryo by the latter route.

Inoculated eggs are sealed with collodion or scotch tape and incubated at the temperature best suited to the growth of the virus. This may range from 35° C. for influenza, 37° C. for psittacosis, or 38° C. for the rinderpest virus. Eggs that die within 24 to 48 hours are usually discarded and considered contaminated. The virus of equine influenza is able to kill 95 per cent of the eggs in 18 hours and this must be taken into consideration. Eggs that die after 48 hours are stored in the ice box. When 50 per cent of the eggs have died, the membranes, extra-embryonic fluids, and embryos of both living and dead embryos are harvested. The tissues and fluids are stored in a deep freeze and can be used for further egg inoculations or for animal experimentation.

The susceptible experimental animal has been widely used in studying viruses. Often, the animal used is expensive and awkward to handle as in the case of the monkey used in studying poliomyelitis, the cow for rinderpest, and the pig for hog cholera. Fortunately most viruses are infective for either the chicken, rabbit, guinea pig, mouse, rat or hamster.

A recent approach taken by investigators has been the adaptation of a virus to a resistant animal by alternate animal passage. Baker (2) demonstrated this technic and pointed out its significance. He was able to establish and maintain in serial passage the virus of rinderpest in rabbits. The virus was alternately transferred from its natural host, the cow, to a rabbit and then back again to a calf. In the passage, the virulence of the virus was increased for the rabbit and clinical symptoms were observed. A similar result was obtained with the virus of hog cholera, alternation being made between pigs and rabbits.

### Serial Passage

A related technic, that of serial passage in one animal has been used to attenuate the virus. Often the 2 technics are combined. The virus of yellow fever has been attenuated by passing the virus from the monkey to mice. By carrying the virus in serial transfer through many generations of mice, the virus become highly virulent for the mice but avirulent for the monkey and man. During the serial passage, a virus is often carried blindly, a febrile response in the infected animal being the only indication as to when the virus should be transferred. Occasionally a virus may be lost in serial passage if the febrile reaction is missed.

In vivo and in vitro tests are used to identify unknown viruses, detect immune bodies in animals, and to determine the potency of a vaccine. The in vivo animal protection and neutralization tests are used to demonstrate cellular immunity. The protection test is carried out by immunizing an animal with suspect serum or vaccine to be tested. Following an interval of a week after the last immunizing injection, the animal is given a dose of virulent virus. Survival of the animal to

this challenge indicates protection by the serum or vaccine. The reaction is highly specific. In the tissue or serum neutralization test, dilutions of known virus are mixed with an equal quantity of suspect or test serum and incubated. This mixture is inoculated into susceptible experimental animals. Absence of clinical symptoms of disease in the test animals plus their susceptibility to a subsequent challenge with virulent virus determines neutralization. The reaction is not as specific as the protection test.

### Complement Fixation Test

The complement-fixation test for virus diseases is carried out in the same manner as with bacteria. It is important in all in vitro tests to note that the titer is of little value in interpreting the test. The titer curve formed from samples of serum obtained during the acute stage and convalescent stage of the disease and run at the same time are of the utmost importance. The cold agglutination test is of use for the diagnosis of virus pneumonia. Suspect serum is mixed with human type "O" red blood cells and allowed to stand overnight in an icebox. A positive reaction is one in which the red blood cells are agglutinated or clumped together after 12 hours in the icebox but which are resuspended when brought to 37° C. for 10 minutes. The agglutination-inhibition test is used for diagnosing influenza. The test employs human type "O" red blood cells although chicken red blood cells also can be used. The test may be run by diluting the serum to be tested and keeping the virus constant or by diluting the virus and keeping the serum constant. A positive serum will inhibit the clumping of red blood cells by the virus. A significant rise in antibody titer is one in which the convalescent serum inhibits at least a 4 fold greater dilution than the acute serum.

The roller tube technic (3) has been developed in an effort to grow viruses in a tissue culture fluid that can be removed and replaced at will over a considerable period of time. Sterile tubes are coated with a film of heparinized chicken plasma. Minced chick embryo and Simms fluid are added. Nutrient fluid containing virus is then added. The nutrient fluid is changed every 48 hours for 15 days until the titer of the virus is $10^{-5}$. The technic may be of value in the production of large quantities of virus and may be used in vaccine production.

Lyophilization is widely used for the preservation of viruses. The virus is suspended in serum or milk and frozen rapidly in a mixture of dry ice and methyl alcohol. The frozen virus is then rapidly dried in a vacuum. This reduces the volume and enables one to store viruses for long periods of time in dry ice without losing virulence or showing a decrease in titer.

The electron microscope is being used to study the particulate nature of the viruses. Measurements made with the electron microscope agree with those made with the Elford ultra filter and the ultra centrifuge. The electron microscope consists essentially of a series of magnets which replace the lens system and which use a stream of electrons as a light source.

Elford's ultra filter is used in measuring the size of viruses. The membrane used for filtration is Shirring's collodion in water and acetic acid. The porosity of the membrane can be varied as little as 10 millimicrons by varying the amounts of acetic acid or water.

The high speed vacuum centrifuge has been used to predict the size and nature of viruses. If a virus is not uniform, the particles will settle out unevenly and nothing can be told of the nature of the virus particles. It has been found that particles of 0.1 to 0.2 microns are thrown down at 10,000 r.p.m. for 30 minutes whereas particles of 60 millimicrons are thrown down at 40,000 r.p.m. for 60 to 90 minutes. The centrifuge has also proven to be of value in the purification of viruses.

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